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Abstract.

Purpose. The aim of this study is to assess the effect of PTH on Wnt10b production by immune system cells in humans. We assessed both the effect of intermittent PTH administration (iPTH) and of chronic PTH hyper secretion in primary hyperparathyroidism (PHP).

Methods. Eighty-two women affected by post-menopausal osteoporosis were randomly assigned to treatment with calcium and vitamin D alone (22) or plus 1-84 PTH (42), or intra venous ibandronate (18). Wnt10b production by unfractioned blood nucleated cells and by T, B cells and monocytes was assessed by real time RT-PCR and ELISA at baseline, 3, 6, 12 and 18 months of treatment.

The effect of chronic elevation of PTH was evaluated in 20 patients affected by PHP at diagnosis and after surgical removal of parathyroid adenoma.

WNT10b from both osteoporotic and PHP patients was compared to healthy subjects matched for age and sex.

Results. iPTH increases Wnt10b production by T cells, whereas PHP does not. After surgical restoration of normal parathyroid function, WNT10b decreases, although it is still comparable with healthy subjects level. Thus chronic elevation of PTH does not significantly increase WNT10b production as respect to control.

Conclusions. This is the first work showing the effect of both intermittent and chronic PTH increase on Wnt10b production by immune system cells. We suggest that, in humans, T cells amplified the anabolic effect of PTH on bone, by increasing Wnt10b production, which stimulates osteoblast activity.

Key words: osteoporosis, primary hyperparathyroidism, PTH, Wnt10b, T cells, immune system.

Mini Abstract.

We evaluated the effect of PTH on Wnt10b production by immune system cells in humans. We showed that bone anabolic effect of intermittent PTH treatment may be amplified by T cells through increased production of Wnt10b. Chronic increase in PTH as in primary hyperparathyroidism does not increase Wnt10b expression.

INTRODUCTION.

Parathyroid hormone (PTH) regulates calcium and phosphate homeostasis and has profound effects on bone turnover. PTH chronic increase, as in primary or secondary hyperparathyroidism, has catabolic effects on bone, causes bone loss and increases fracture risk [1]. In contrast, intermittent PTH injection, has anabolic effects on bone [2] and prevents fragility fractures, indeed intermittent PTH is currently used for the treatment of post-menopausal osteoporosis [3,4].

The PTH receptor (PPR) is mainly expressed in bone and kidney, but its expression has also been reported in other tissues where it likely reflects the local paracrine role of PTH related protein [5-7]. In bone, PPR is expressed in osteoclasts [8] and mainly in cells of osteoblastic lineage as osteocytes [9]. A recent work by Saini et al [9] suggests that osteocytes are necessary for intermittent PTH anabolic effects. Treatment with intermittent PTH activates Wnt signalling pathway in osteoblast by suppressing Sclerostin production by osteocytes [10-16].

PPR is also expressed by T cells [17], which are required for intermittent PTH to exert its bone anabolic effect as recently demonstrated in mice [17-19]. The bone anabolic response to intermittent PTH is blunted in the absence of T cells and is restored by adoptive transfer of these cells [17,18]. T cells mediate intermittent PTH anabolic effect on bone through the up-regulation of Wnt10b on their surface [18]; Wnt10b interacts with osteoblasts up-regulating Wnt pathway, and induces bone formation.

Unlike intermittent PTH, continuous PTH infusion, a condition that mimics primary hyperparathyroidism (PHP) in humans, doesn't affect Wnt10b expression by T cells in mice [17,18], whereas in humans no data are available.

Here we show that PTH affects Wnt10b production by immune system cells in humans, potentially explaining its anabolic effect.

MATERIALS AND METHODS

Patients.

The study was approved by the Ethical Committee of the A.O.U. Città della Salute e della Scienza - A.O. Ordine Mauriziano - A.S.L. TO1, Turin Italy and informed consent was obtained from all participants. The study population was recruited from the patients of A.O.U. Città della Salute e della Scienza, Turin Italy and healthy volunteers. The study included patients affected by post-menopausal osteoporosis and patients affected by primary hyperparathyroidism (PHP).

Post-menopausal osteoporosis.

Eighty-two women affected by post-menopausal osteoporosis without fractures were randomly allocated to treatment with:

i. intermittent PTH (PTH 1-84 100 mcg/day s.c. -Preotact ®, kindly provided by Nycomed-plus calcium 1200 mg/day and colecalcipherol 800 UI/day, referred to hereafter as iPTH), 42 patients,
ii. calcium and vitamin D 1200 mg/day and colecalcipherol 800 UI/day (referred to hereafter as calcium and vitamin D), 22 patients
iii. intravenous ibandronate 3mg every 3 months, plus calcium and vitamin D (referred to hereafter as IB), 18 patients.

This is a parallel, randomized controlled, open label, trial (registered as PTH1-84 EudraCT 2009-012397-12). The randomization was done by computer generated tables to allocate treatments. Randomization was done by the principal investigator, patients were enrolled by participants in the study, lab measurement and statistical analyses were done in blind to treatment.

Patients affected by secondary osteoporosis or taking drugs active on bone metabolism were not considered eligible for the study.

Blood samples from patients were collected at baseline and after 3, 6, 12 and 18 months of treatment.

Primary hyperparathyroidism.

We enrolled in the study 20 patients (16 women and 4 men) affected by PHP, none of them were affected by diseases affecting bone health other than PHP. Subjects with secondary hyperparathyroidism, severe vitamin D deficiency, chronic renal disease (GFR<60) and any other condition known to affect PTH levels were excluded.

The diagnosis of PHP was established on the finding of elevated circulating levels of calcium and PTH in at least 2 instances and the presence of normal renal function. PHP patients were subjected to parathyroidectomy and restoration of normal parathyroid function was demonstrated by the finding of normal serum PTH and calcium levels 1 month after surgery. Blood samples were collected at baseline and 1 months after parathyroidectomy.

Healthy controls.

Two groups of healthy subjects were enrolled as controls for post-menopausal women (31 healthy post-menopausal women) and for PHP patients (42 healthy men and women in post-menopausal period or in fertile age). Patients and controls were matched for sex, age and years since menopause.

The study design is summarized in figure 1. The demographic characteristics of the study population are shown in table 1.

Measurements of Wnt 10b mRNA.

Wnt10b mRNA was measured in unfractioned peripheral blood nucleated cells in osteoporotic patients, in PHP and in healthy controls. Unfractioned peripheral blood nucleated cells were obtained by red blood cells lyses, the obtained cells were frozen at -80°C until RNA extraction. In 36 osteoporotic patients (18 treated with iPTH and 18 with IB) T cells, B cells and monocytes were separated by immunomagnetic beads separation (Stemcells Technology) to evaluate which cell produces Wnt10b (Fig.1).

Real-Time PCR (RT-PCR) was used to evaluate Wnt10b expression. RNA was isolated using TRIzol reagent (Ambion, Huntingdon, UK), chloroform extraction, and subsequent isopropanol precipitation according to the manufacturer's protocol. One µg of RNA was converted up to single-stranded cDNA by the High Capacity cDNA Reverse Transcription Kit (Applied-Biosystems). RT-PCR was performed with IQ SYBR Green Supermix (BIORAD). The housekeeping control gene was β-Actin and gene expression was quantified through 2-ΔΔCt method. The primers used were:
5'- CCATGACATGGACTTTGGAGAG -3' (forward), 5'- CTGGAATCCAAGAAATCCCG -3' (reverse) for Wnt10b and 5'- CCTAAAAGCCACCCCACTTCT -3' (forward) and 5'- CACCTCCCCTGTGTGGACTT -3' (reverse) for β-Actin.

Protein detection.

Wnt10b protein was measured on cell lysates by ELISA technique (USCN Life Science) after correction for total amount of protein.

Statistical analyses.

Wnt10b values were not normally distributed according to kurtosis normality test, hence the effect of treatment on its expression was evaluated by repeated measure tests, after logarithmic transformation. Wnt10b mRNA levels were analysed by Mann Whitney (healthy

controls vs. osteoporotic or PHP) and Wilcoxon matched pairs signed rank tests (PHP vs. PHP after surgery and different cell types).

The sample size provided an 80% power, assuming a two-sided significance level of 0.05, to detect differences in Wnt10b of 3 fold, according with the results obtained in mice [18].

The statistical analyses was performed through SPSS 21.0 and graphs were designed through Prism Graph Pad 6.0. .

RESULTS.

Osteoporosis does not affect Wnt10b production by blood nucleated cells.

To evaluate whether Wnt10b expression was decreased in osteoporosis, we compared its expression in unfractioned blood nucleated cells from osteoporotic patients and healthy women, matched for age and post-menopausal period. Wnt10b was not significantly different between patients and controls (Fig.2 A), suggesting that it is not involved in the pathogenesis of post-menopausal osteoporosis.

iPTH increases Wnt10b production by T cells.

Treatment with iPTH in osteoporotic women increases Wnt10b gene expression in peripheral blood nucleated cells, whereas calcium and vitamin D alone do not (Fig.2B). In particular Wnt10b increases of approximately 21 fold after 6 months of treatment and returned to baseline values after 18 months (Fig.2B).

Wnt10b protein level, detected by ELISA, confirmed an increase of Wnt10b production during treatment with iPTH (Fig.2C), according to the RT-PCR results. The increase in Wnt10b mimics the observed rise in bone alkaline phosphatase (BAP, Fig.2D), that is a well-known bone formation marker.

The analyses of separated T, B cells and monocytes as compared to unfractioned blood nucleated cells revealed that T cells are the main responsible for Wnt10b expression in

osteoporotic patients without treatment, whereas B cells and monocytes only express a small amount of this molecule (Fig. 3 A). Further evaluation of T cells during iPTH treatment compared to IB reveals that the increase of Wnt10b expression depend on iPTH, indeed IB does not induce any significant variation (Fig 3 B). B cells and monocytes do not increase Wnt10b expression during treatment (Fig. 3 C and D).

Chronic elevation of PTH did not increase Wnt10b expression.

Wnt10b was not increased in unfractioned peripheral blood nucleated cells from patients affected by PHP compared to healthy controls (Fig. 4), nevertheless surgical restoration of normal parathyroid function decreased Wnt10b expression of about 36%.

Even though surgical intervention decreased Wnt10b, its expression remain not significantly different as respect to healthy controls (Fig.4).

All the surgical intervention were completely successful as demonstrated by the fall in PTH level from 136±26 ng/mL to 68±8 ng/mL, p=0.008.

DISCUSSION

This study explores the effect of iPTH and PHP on Wnt10b production by T cells in humans we show that T cells are the main producers of Wnt10b amongst peripheral blood nucleated cells, and Wnt10b expression increases during iPTH treatment. These data suggest that T cells may mediate the anabolic action of iPTH in humans as they do in mice [17,18]. According to this hypothesis, literature data derived from animal models report that the anabolic activity of iPTH depends on T cells, which increase the expression of Wnt10b. [17,18]. Indeed, treatment with iPTH in mice stimulates T cell production of Wnt10b, that increases osteoblastogenesis. T cell-deficient mice or mice with T cell-knock out for Wnt10b display a blunted bone anabolism after treatment with iPTH [17].

The analysis of Wnt10b expression in peripheral blood nucleated cells, at different time during iPTH treatment, allows us to create a Wnt10b curve that reveals an increase in its expression that is maximal 6 months after treatment. This increase was not observed in patients treated with calcium and vitamin D alone. The Wnt10b curve in response to iPTH mimics the well-known bone anabolic markers curve [3,4], confirming Wnt10b role in mediating iPTH anabolic action.

Differently from iPTH, chronic elevation of PTH, as in PHP, does not increase Wnt10b expression. However, one month after surgical intervention, Wnt10b expression results significantly decreased, but comparable to healthy subjects. This result may depend on the small size of the cohort analyzed, but we speculate a possible effect of chronic elevation in PTH on Wnt10b that is not sufficient to increase it above normal range. To support this hypothesis, literature data report that chronic elevation of PTH in PHP modulates Wnt signaling pathway also by suppressing SOST in humans [20, 21] as well as in mice [22].

This observation may explain the anabolic effect on trabecular bone of PHP, indeed PHP preferentially involves cortical bone with preservation of cancellous areas, as demonstrated by histomorphometric analysis. In particular, the majority of patients with PHP showed reductions in cortical width, whereas the cancellous compartment of the bone biopsy specimen showed greater than average values for trabecular bone volume, trabecular number, connectivity and separation, indicating preservation of this bone compartment in most patients with PHP [23-26]. Here we describe a decrease in Wnt10b, after surgical restoration of normal parathyroid function, which may partially explain the anabolic effect of PHP on trabecular bone.

Our study has a number of strengths: to our knowledge, this work represents the first study in humans to evaluate the effect of PTH on peripheral blood nucleated cells with particular regards to T cells. In addition, we attempted to do so without in vitro culture of the cells, which could substantially alter their gene expression and other characteristics. Patients

and controls have been carefully matched for potential confounders and randomized to different treatment group. However one major limitation of our work is the small sample size especially of PHP cohort.

In conclusion this study reports that PTH induces an increase of Wnt10b production by T cells in humans. Thus, our data suggest that T cells amplify the anabolic effect of PTH on bone.

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Table 1. A Characteristics of osteoporotic patients and healthy controls.

B Characteristics of PHP and healthy controls.

Mean and standard deviations are shown for continuous variables, % for non-continuous one. P values were calculated by ANOVA one-way for continuous variable and by $\chi 2$ for non-continuous one.

A	**Post-menopausal osteoporosis**				
	Patients			**Controls**	**p value**
Treatment	iPTH	Calcium and vitamin D	IB	none	
Patients (n)	42	22	18	31	
Women (%)	100%	100%	100%	100%	
Men (%)	0%	0%	0%	0%	
Age (yrs)	68.1±9.5	66.6±6.2	64.7±9.2	69±15.2	0.263
Years since menopause	18.6±10	14.8±9.3	17.7±10	17.0±10.6	0.186

B	**PHP**		
	PHP	**Controls**	**p value**
Patients (n)	20	42	
Women (%)	80	70	0.366
Women in after menopause (%)	56	47	0.191
Men (%)	20	40	0.366
Age (yrs)	57.3±15.4	50.5±21.5	0.214
Years since menopause	16±9.4	20.5±14.7	0.400

FIGURES.

Figure 1. The diagram shows the study design: the number of patients in each group and at each visit and the experiments done are specified.

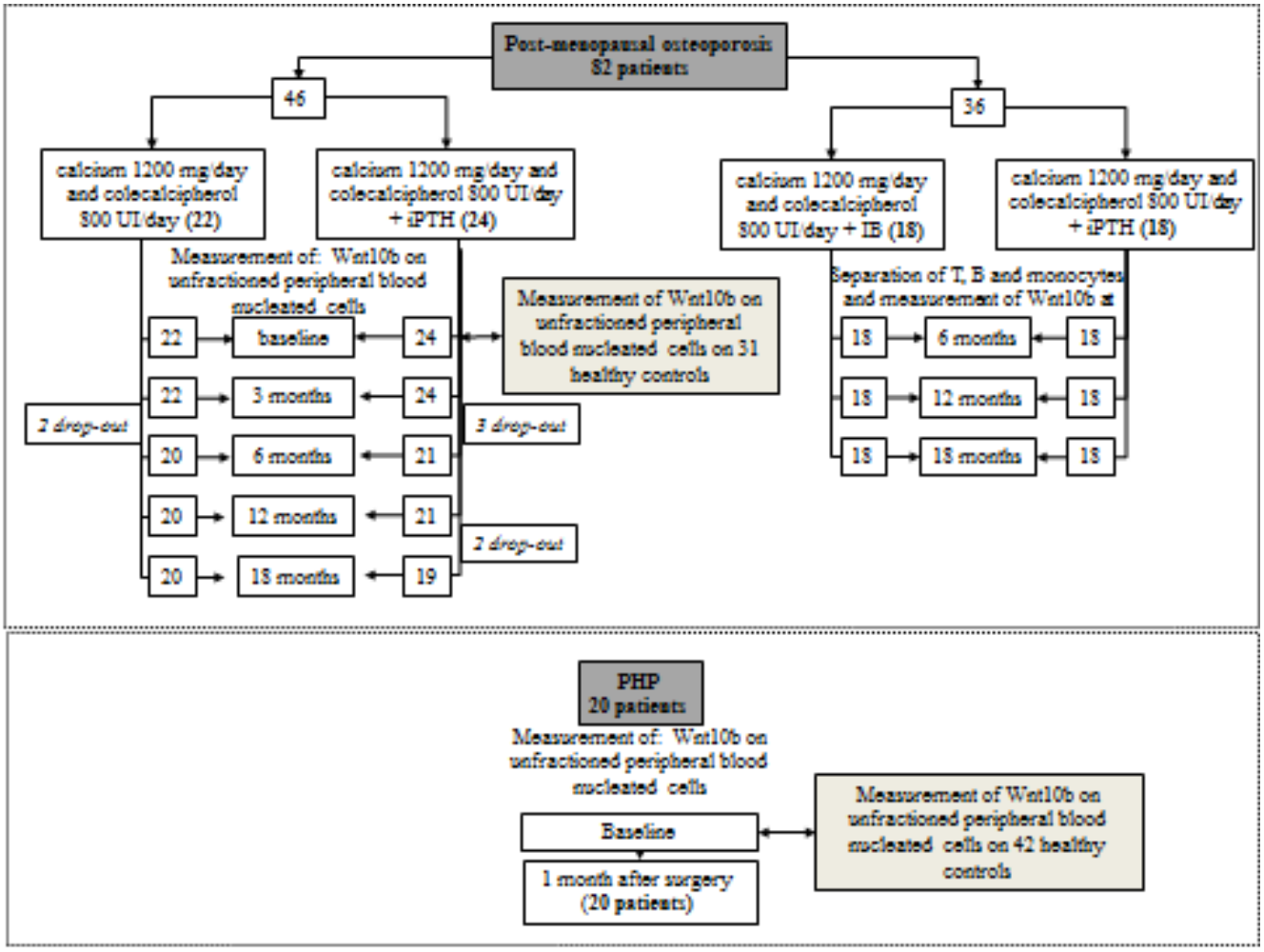

Figure 2. Wnt 10b expression in osteoporosis and during treatment.

A. Levels (Median mean ± SE) of Wnt10b mRNA in healthy controls (n = 31) and osteoporotic subjects (n = 46). Data were analysed by Mann Whitney as the data were not normally distributed according to the kurtosis normality test.

B. Wnt10b mRNA fold change versus baseline in osteoporotic patients treated with iPTH (n=21) or calcium and vitamin D (n=20). Data were analysed by multiple measurement test after logarithmic transformation, as the data were not normally distributed according to the kurtosis normality test.

C. Wnt10b protein production by unfractioned blood nucleated cells in osteoporotic patients treated with iPTH (n=21) or calcium and vitamin D (n=20). Data were analysed by Multiple measurement test after logarithmic transformation, as the data were not normally distributed according to the kurtosis normality test.

D. Serum BAP in osteoporotic patients treated with iPTH (n=21) or calcium and vitamin D (n=20). Data were analysed by multiple measurement test.

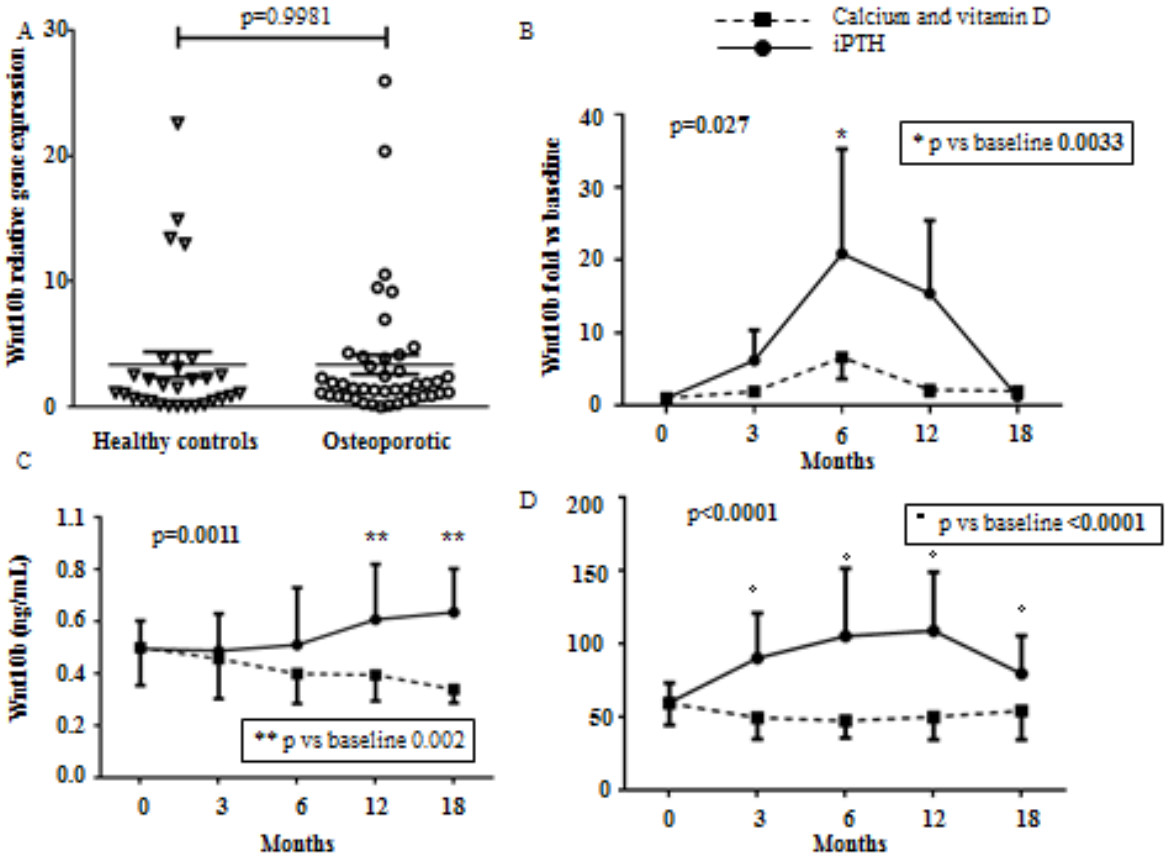

Figure 3. Wnt 10b expression by T cells.

A. Wnt10b mRNA expression relative to unfractioned blood nucleated cells (median mean ± SE) in monocytes, T and B cells from osteoporotic patients without treatment (n = 36). Data were analysed by Wilcoxon matched pairs signed rank test as the data were not normally distributed according to the kurtosis normality test.

B. T cells Wnt10b mRNA fold change versus baseline in osteoporotic patients treated with iPTH (n=18) or IB (n=18). Data were analysed by multiple measurement test after logarithmic transformation, as the data were not normally distributed according to the kurtosis normality test.

C. B cells Wnt10b mRNA fold change versus baseline in osteoporotic patients treated with iPTH (n=18) or IB (n=18). Data were analysed by multiple measurement test after logarithmic transformation, as the data were not normally distributed according to the kurtosis normality test.

D. Monocytes Wnt10b mRNA fold change versus baseline in osteoporotic patients treated with iPTH (n=18) or IB (n=18). Data were analysed by multiple measurement test after logarithmic transformation, as the data were not normally distributed according to the kurtosis normality test.

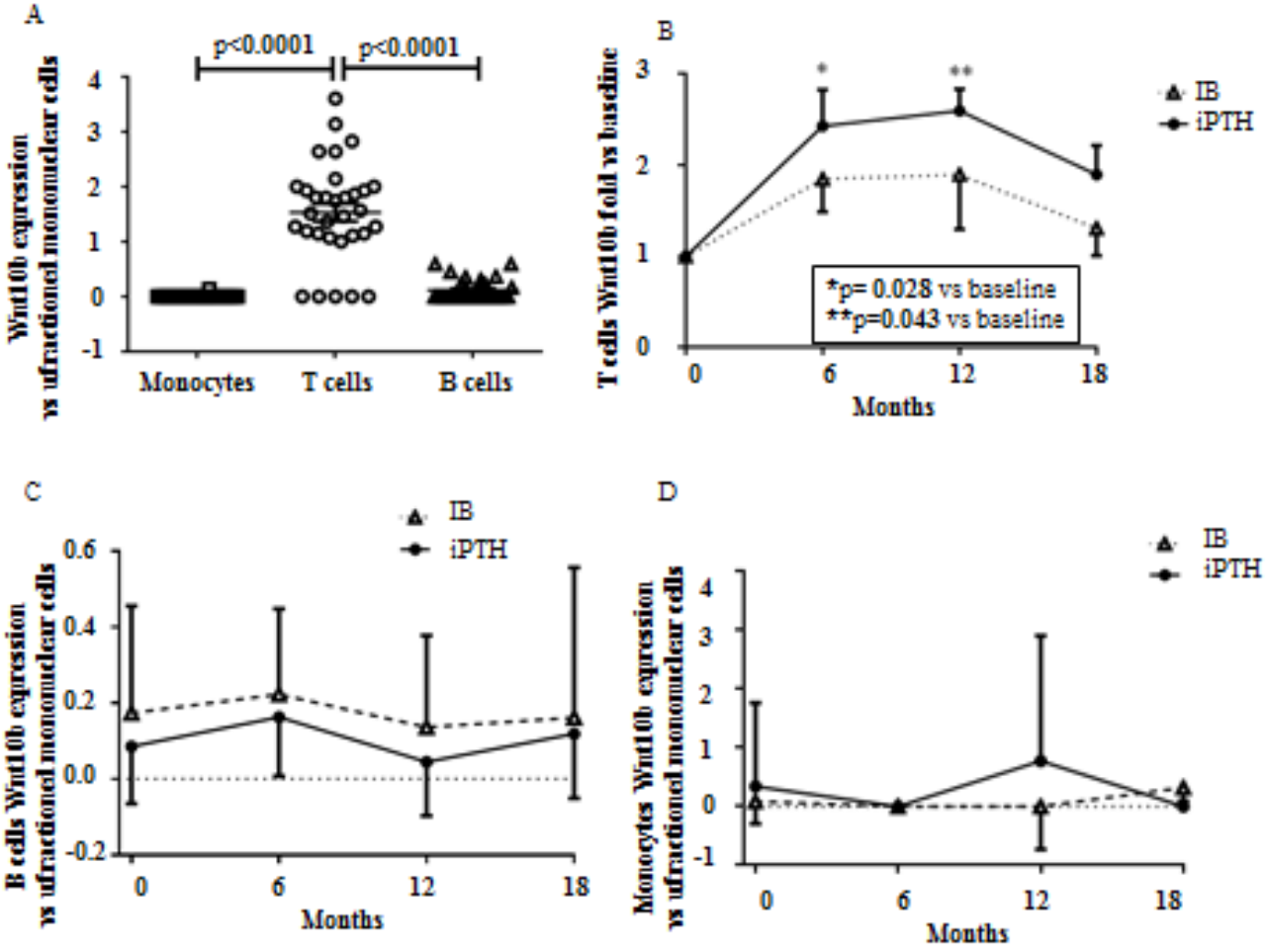

Figure 4. Wnt 10b expression in PHP. Levels (Median mean ± SE) of Wnt10b mRNA in healthy controls (n = 42) and PHP before (n = 20) and after parathyroidectomy (n = 20). Data were analysed by Mann Whitney (healthy controls vs. PHP) and Wilcoxon matched pairs signed rank tests (PHP vs. PHP after surgery) as the data were not normally distributed according to the kurtosis normality test.